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# Reduction of α-Oxo Carboxylic Acids by Pigeon Liver 'Malic' Enzyme

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1. Pigeon liver 'malic' enzyme [L-malate–$NADP^+$ oxidoreductase (decarboxylating); EC 1.1.1.40] was shown to catalyse the reductase reaction:

$$\text{Oxaloacetate} + \text{NADPH} + H^+ \xrightarrow{Mn^{2+} \text{ or } Mg^{2+}} \text{L-malate} + NADP^+$$

L-Malate was identified as the reaction product, and was formed in stoicheiometric amount. 2. In addition to oxaloacetate and pyruvate, a number of other α-oxo carboxylic acids were also reduced.

Pigeon liver 'malic' enzyme [L-malate–$NADP^+$ oxidoreductase (decarboxylating); EC 1.1.1.40] can catalyse the decarboxylation of oxaloacetate (reaction 1), as shown by Ochoa *et al.* (1948):

$$\text{Oxaloacetate} \xrightarrow{Mn^{2+} \text{ or } Mg^{2+}} CO_2 + \text{pyruvate} \quad (1)$$

Attempts by these investigators to demonstrate the reduction of oxaloacetate to L-malate were not successful. More recently, this enzyme was obtained in pure form and shown to catalyse a slow reduction of pyruvate to form L-lactate (reaction 2; Hsu & Lardy, 1967*a*):

$$\text{Pyruvate} + \text{NADPH} + H^+ \xrightarrow{Mn^{2+} \text{ or } Mg^{2+}} \text{L-lactate} + NADP^+ \quad (2)$$

The pigeon liver enzyme, and one partially purified from wheat germ, also oxidized NADPH in the presence of oxaloacetate (Hsu, 1970; Tsai *et al.*, 1971). This activity was thought to be due to reaction (3):

$$\text{Oxaloacetate} + \text{NADPH} + H^+ \xrightarrow{Mn^{2+} \text{ or } Mg^{2+}} \text{L-malate} + NADP^+ \quad (3)$$

The reaction product, L-malate, however, was not identified. Since oxaloacetate may be decarboxylated (reaction 1), yielding pyruvate and $CO_2$ which undergo the reductive carboxylation reaction (4):

$$\text{Pyruvate} + CO_2 + \text{NADPH} + H^+ \xrightarrow{Mn^{2+} \text{ or } Mg^{2+}} \text{L-malate} + NADP^+ \quad (4)$$

the possibility existed that this and reaction (2), rather than reaction (3), were responsible for the oxaloacetate-dependent oxidation of NADPH.

The present report provides conclusive evidence, however, that the 'malic' enzyme catalyses conversion of oxaloacetate into L-malate according to reaction (3). L-Malate was identified by enzymic analysis and was formed in stoicheiometric amounts. In addition to oxaloacetate and pyruvate, a number of α-oxo carboxylic acids are reduced at low rates.

## Materials and Methods

Sources of reagents were as noted here: triethanolamine hydrochloride, oxaloacetic acid, glyoxylic acid monohydrate, sodium 2-oxovalerate, 2-oxo-octanoic acid and 2-oxoisohexanoic acid (Calbiochem, La Jolla, Calif., U.S.A.); sodium phenylpyruvate and 4-ethyloxaloacetate (Nutritional Biochemical Corp., Cleveland, Ohio, U.S.A.); *N*-ethylmaleimide (Sigma Chemical Co., St. Louis, Mo., U.S.A.); $NAD^+$, NADH, $NADP^+$ and NADPH (P-L Biochemicals Inc., Milwaukee, Wisc., U.S.A.); malate dehydrogenase (Boehringer Mannheim Corp., New York, N.Y., U.S.A.) were purchased. Distilled deionized water was used.

Pigeon liver malic enzyme was purified by the method of Hsu & Lardy (1967*b*). The specific activity for oxidative decarboxylation at 30°C was 40μmol/min per mg of protein. Each preparation was examined in the ultracentrifuge for homogeneity. A modified enzyme was prepared by incubating the enzyme with *N*-ethylmaleimide at room temperature (Table 1, also Tang & Hsu, 1972). All enzyme assays were performed with a Cary model 16 spectrophotometer equipped with a Cary model 1626 recorder interface and a Hewlett Packard model 7107B recorder. The cell compartment was at 30°C. The formation (or disappearance) of NADPH was followed at 340nm. The oxidative decarboxylase activity was determined by the method of Hsu & Lardy (1967*b*). The reaction mixture for the standard reductase assay contained: triethanolamine–HCl buffer, pH7.4, 267mM; NADPH, 0.21mM; $MgCl_2$, 4mM; oxaloacetate, 6.67mM; enzyme; total volume 3.0ml. Oxaloacetic acid was dissolved in water and used within

30min. The reaction rate was constant for 3–4min and was proportional to enzyme concentration up to 10$\mu$g/assay. One unit of enzyme activity is defined as the formation (or disappearance) of 1$\mu$mol of NADPH/min. Specific activity is expressed in units/mg of protein.

## Results

Table 1 shows the NADPH- and metal-dependent reduction of oxaloacetate to L-malate. Corrections were made for L-malate formed by malate dehydrogenase activity (in the absence of $Mg^{2+}$), and by the reductive carboxylation (reaction 4) activity, by using pyruvate and $CO_2$ at concentrations equal to those obtainable after complete decarboxylation of the oxaloacetate. A net value of 0.031$\mu$mol of L-malate was found in Expt. 1. This value, although much lower than expected on the basis of initial-velocity measurements, represents the minimum amount of L-malate synthesized through the direct reduction of oxaloacetate according to reaction (3). Other experiments with more enzyme failed to increase the yield of L-malate, because of the potent oxidative decarboxylase activity, which removed L-malate as soon as it was formed. Therefore 'malic' enzyme treated with *N*-ethylmaleimide was used in Expt. 2. The modified enzyme is suited for this purpose, since it is completely devoid of the oxidative decarboxylase activity in both directions but still acts as an oxaloacetate or pyruvate reductase (C. L. Tang & R. Y. Hsu, unpublished work). Strong evidence for the direct reduction of oxaloacetate to L-malate via reaction (3) is seen. The amount of NADPH oxidation was slightly higher than the expected 1:1 stoicheiometry, because of the enhanced ability of the modified enzyme to reduce pyruvate (product of reaction 1) to L-lactate (reaction 2). Reaction (2) also accounts for the NADPH oxidation (but not L-malate formation) in the pyruvate plus $NaHCO_3$ control (Expt. 2B). The malate dehydrogenase control value was negligible, as expected.

Other experiments showed that oxaloacetate reduction is dependent upon the presence of NADPH, NADH being completely ineffective, and either $Mg^{2+}$ or $Mn^{2+}$. Cofactor requirements for the reduction of other substrates such as mesoxalate and 2-oxoglutarate were identical. $Mn^{2+}$ was effective at a concentration one order of magnitude lower than $Mg^{2+}$, but at concentrations above 1mM inhibited the reduction of oxaloacetate. The pH–activity relationship was determined from pH5.6 to 8.2 in the standard assay system (oxaloacetate concentration, 5.33mM) in triethanolamine buffer adjusted to the desired pH values; the optimum pH was 6.5.

Fig. 1 shows double-reciprocal plots of reaction rate versus oxaloacetate concentration. The apparent Michaelis constants of oxaloacetate in the $Mg^{2+}$ (4mM)-activated reaction catalysed by the native and *N*-ethylmaleimide-modified enzyme were 10mM and 8.3mM respectively. When $Mn^{2+}$ (0.2mM) was used as the activator in the reaction catalysed by the untreated enzyme, a value of 3.3mM was obtained.

Oxaloacetate is a substrate for the decarboxylation reaction (reaction 1) as well as an inhibitor of the oxidative decarboxylation of L-malate (Stickland,

Table 1. *Reduction of oxaloacetate to* L-*malate by 'malic' enzyme*

Complete assays (A) contained: oxaloacetate, 1.33mM; NADPH, 0.21mM; triethanolamine–HCl buffer, pH7.4, 67 or 267mM; $MgCl_2$, 2 or 4mM; native enzyme, 90$\mu$g, or enzyme-treated with *N*-ethylmaleimide, 150$\mu$g, in Expts. 1 and 2 respectively. The reactions were carried out at 26°C in a total volume of 3.0ml. After 30min, the extent of NADPH oxidation was determined at 340nm. The reaction was stopped by the addition of 0.15ml of 70% perchloric acid, and the mixture was neutralized with 0.3ml of 5M-$K_2CO_3$. A sample of the supernatant solution was analysed for L-malate by the method of Hohorst (1965). The modified enzyme was prepared by incubating 1.89mg of enzyme with 0.3$\mu$mol of *N*-ethylmaleimide at pH7.0 (total volume, 0.63ml) for 4h at room temperature.

| | Expt. 1 (native enzyme) | | Expt. 2 (modified enzyme) | |
|---|---|---|---|---|
| Assay treatment | NADPH oxidized ($\mu$mol) | L-Malate found ($\mu$mol) | NADPH oxidized ($\mu$mol) | L-Malate found ($\mu$mol) |
| (A) Complete | 0.162 | 0.091 | 0.338 | 0.294 |
| (B) −Oxaloacetate, +pyruvate and $NaHCO_3$ (1.33mM each) | 0.062 | 0.035 | 0.378 | 0.010 |
| (C) −$MgCl_2$, +EDTA (33mM) | 0.025 | 0.025 | 0.004 | 0.020 |

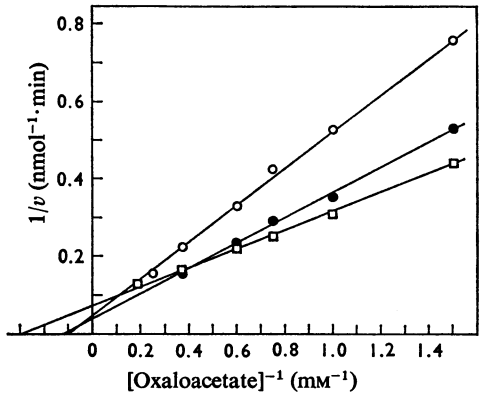


Fig. 1. *Lineweaver–Burk plot of reductase activity versus oxaloacetate concentration*

Reduction assays were carried out as described in the Materials and Methods section except that either $Mg^{2+}$ (4mM; ○, ●) or $Mn^{2+}$ (0.2mM; □) was used, and the concentration of oxaloacetate was varied as indicated. Each cuvette contained 10.5$\mu$g of native enzyme (○, □) or 26.0$\mu$g of *N*-ethylmaleimide-modified enzyme (●).

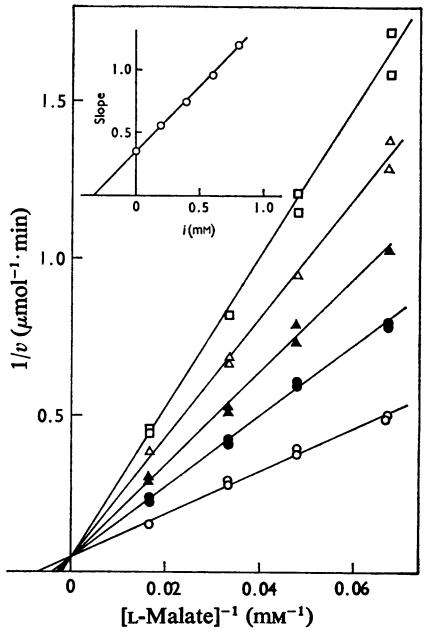


Fig. 2. *Inhibition of the oxidative decarboxylation reaction by oxaloacetate, with* L-*malate as the variable substrate*

Reaction mixtures contained: triethanolamine–HCl buffer, pH7.4, 67mM; $MgCl_2$, 4mM; $NADP^+$, 0.23mM; purified 'malic' enzyme, 1.42$\mu$g; various amounts of L-malate and oxaloacetate as indicated. Total volume, 3.0ml. Temperature, 30°C. Oxaloacetate concentrations used were (mM): ○, 0; ●, 0.2; ▲, 0.4; △, 0.6; □, 0.8. Plots were drawn by least-squares fitting of experimental points to a straight line. Inset: replot of slopes versus oxaloacetate concentration (*i*).

1959); this inhibition is competitive with respect to L-malate (Fig. 2). The Michaelis constant of L-malate at pH7.4 was 0.167mM in the absence of oxaloacetate, and increased to a value of 0.556mM at an oxaloacetate concentration of 0.8mM. An inhibition constant ($K_i$) of 0.32mM was determined graphically (Fig. 2 insert).

The substrate specificity of the reductase reaction is shown in Table 2. Except for 2-oxoisovaleric acid, all other α-oxo mono- and di-carboxylic acids tested were reduced by the native enzyme at measurable ..tes. Among these compounds, oxaloacetate exhibited the highest turnover rate (i.e. a specific activity of 0.6–0.7), which was approx. 2% of the oxidative decarboxylase activity at the same pH. 4-Ethyloxaloacetate, which is not a substrate for decarboxylation because of its blocked carboxyl group (Tsai *et al.*, 1971), was nevertheless reduced at a slow rate. Under these conditions, no pyruvate was available to accept reducing equivalents from NADPH through either the reductive carboxylation (reaction 4) or reduction (reaction 2) reactions. Therefore reduction of 4-ethyloxaloacetate must occur through a one-step reaction analogous to reaction (3), resulting in the formation of 4-ethylmalate as the product.

Further, the rate of NADPH oxidation in the presence of both oxaloacetate and its 4-ethyl derivative was significantly lower than the sum of individual rates, indicating that these two substrates were competing for the same catalytic site for reduction.

Table 2 also shows the reductase activity of the *N*-ethylmaleimide-modified enzyme on various α-oxocarboxylic acids. The reduction rates of all substrates tested, with the exception of oxaloacetate and 2-oxoglutarate, were enhanced by reaction with *N*-ethylmaleimide.

## Discussion

Results presented in this study provide conclusive evidence that reduction occurs via reaction (3), in which oxaloacetate is reduced by purified 'malic' enzyme to give L-malate. The product of this reaction was identified as L-malate by a specific enzymic assay

Table 2. *Substrate specificity of the reductase reaction*

The standard reductase assay was used, except that oxaloacetate was substituted by other α-oxo carboxylic acids as indicated. Modified enzyme was prepared essentially as in Table 1, except that 3.12mg of purified enzyme (specific activity for oxidative decarboxylase, 40) was incubated with 0.2μmol of *N*-ethylmaleimide in a volume of 1.0ml for 3h.

| Substrate | Specific activity (μmol of NADPH/min per mg of protein) | |
|---|---|---|
| | Native enzyme | Modified enzyme |
| Oxaloacetate (5.33mM) | 0.701 | 0.596 |
| 4-Ethyloxaloacetate (4.17mM) | 0.0361 | 0.0807 |
| Oxaloacetate (5.33mM) + 4-ethyloxaloacetate (4.17mM) | 0.230 | — |
| 2-Oxoglutarate (8.3mM) | 0.034 | 0.028 |
| Glyoxylate (8.3mM) | 0.032 | 0.042 |
| Mesoxalate (8.3mM) | 0.050 | 0.081 |
| Pyruvate (6.7mM) | 0.340 | 1.00 |
| 2-Oxobutyrate (8.3mM) | 0.021 | 0.186 |
| 2-Oxovalerate (8.3mM) | 0.017 | 0.019 |
| 2-Oxoisohexanoate (6.4mM) | 0.0031 | — |
| 2-Oxo-octanoate (5.3mM) | 0.0054 | — |
| Phenylpyruvate (1.11mM) | 0.0015 | — |
| 2-Oxoisovalerate (8.3mM) | 0 | — |

for L-malate. A 1:1 stoicheiometry of L-malate formation and NADPH oxidation was also observed. This reaction specifically requires NADPH (but not NADH), and either $Mg^{2+}$ or $Mn^{2+}$ as a metal activator. The properties of metal activation are similar to those observed on the oxidative decarboxylation of L-malate (Tsai *et al.*, 1971; Stickland, 1959). At low concentrations, the $Mn^{2+}$-activated reduction reaction is more efficient, as seen by the higher turnover rate and lower $K_m$ of oxaloacetate. At higher concentrations, however, $Mn^{2+}$ becomes inhibitory. The pH optimum for oxaloacetate reduction was 6.5, which was identical with that reported for the reduction of pyruvate (Hsu & Lardy, 1967*a*).

The substrate specificity of the reductase reaction is in accord with the structural features required by this enzyme; of all compounds examined, oxaloacetate and pyruvate are most reactive. The relatively low reduction rates of all α-oxo carboxylic acids, including oxaloacetate and pyruvate, as compared with the oxidative decarboxylase activity of the native enzyme, may explain the failure by other investigators to observe the reduction of oxaloacetate by the pigeon liver enzyme (Ochoa *et al.*, 1948) or the reduction of glyoxylate by the 'malic' enzyme isolated from *Pseudomonas* (Cazzulo & Massarini, 1972).

α-Oxo carboxylic acids have been reported to be inhibitors of the oxidative decarboxylase reaction (Stickland, 1959; Zink, 1972; Cazzulo & Massarini, 1972).

Further examination of the reaction rates of various α-oxo carboxylic acid substrates reveal the following trends. (1) Except for mesoxalate, the reaction rate decreases with increasing chain length in either the mono- or di-carboxylic acid homologous series; (2) reaction rates of dicarboxylic acids are higher than those of monocarboxylic acids containing the same number of carbon atoms; (3) substitution of a hydrogen atom by a methyl group at the β-position of α-oxo monocarboxylic acid is not allowable. Thus 2-oxoisovalerate is completely inactive, whereas both 2-oxovalerate and its higher homologue, 2-oxo-octanoate, are active substrates. Moreover, 2-oxo-isohexanoate, which has a branching point at the γ-position, is also active. These observations, although tentative, are in general agreement with the specificity requirements of the alcohol and lactate dehydrogenases reported in the literature (Eys & Kaplan, 1957; Meister, 1950).

Reaction of thiol groups in 'malic' enzyme with *N*-ethylmaleimide at neutral pH leads to the loss of oxidative decarboxylase activity, and concomitant increase of reductase activity on pyruvate (C. L. Tang & R. Y. Hsu, unpublished work). Table 2 shows the enhancement of the reductase activities on a number of 2-oxo monocarboxylic acids and 4-ethyloxaloacetate. The apparent lack of enhancement on the reduction of dicarboxylic acids, oxaloacetate and 2-oxoglutarate suggests that a charged ω-carboxyl group on the substrate molecule may be an important factor. Moreover, manifestation of the inhibition by this group appears to be dependent upon the length of the carbon chain, and is not seen on the short-chain dicarboxylic acid, mesoxalate.

It is clear from the foregoing discussion and arguments presented in previous publications (Hsu & Lardy, 1967*a*; Hsu, 1970) that the observed reductase activity is an intrinsic property of the 'malic' enzyme. A number of observations also imply a common active site for both the oxidative decarboxylation and reduction reactions. Thus oxaloacetate and pyruvate, the two reactants participating in major reactions (i.e. oxaloacetate decarboxylase and reductive carboxylase reactions), are most active in the reductase reaction. Oxaloacetate, which is reduced at the highest rate, is also a competitive inhibitor of the oxidative decarboxylation of L-malate.

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